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16. ABSTRACT

An initiation/promotion bioassay for chemical carcinogens and tumor promoters has been developed in rat liver using presumed preneoplastic lesions, foci of γ-glutamyltranspeptidase (GGTase)-positive hepatocytes, as the endpoint. To evaluate the tumor-promoting activity of phenobarbital, rats were administered diethylnitrosamine (DENA), 2.0 mmole/kg, followed by 500 ppm phenobarbital in their drinking water. After 6 weeks of phenobarbital promotion, the rats had an increased incidence of foci as compared to nonphenobarbital-treated rats. By 50 weeks, the number of foci in the nonpromoted animals equaled the number observed with promotion. The stability and progression of GGTase-positive foci was determined in rats that received a 2/3 partial hepatectomy, followed 24 hours later by DENA administration (0.3 mmole/kg). The rats then received 500 ppm phenobarbital in the drinking water for 7 weeks. After 7 weeks, half of the rats were continued on phenobarbital and the other half were removed from phenobarbital treatment. The number of foci observed in rats continued on phenobarbital treatment leveled off after 10 weeks of promotion, while in rats taken off phenobarbital it did not regress but increased at a slower rate, and, by 56 weeks, approached the number observed in rats subjected to continuous promotion. At 56 weeks, the size of foci was larger after continuous promotion. At 81 weeks, all 6 (100%) of the rats on continuous promotion had liver tumors, while only 3 of 6 (50%) of the rats removed from promotion had tumors. Promotion by phenobarbital stimulated the growth and decreased the time required for the appearance of GGTase-positive foci and liver tumors.

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Tumor Promotion in Rat Liver

by Sydna L. Herren* and Michael A. Pereira*

An initiation/promotion bioassay for chemical carcinogens and tumor promoters has been developed in rat liver using presumed preneoplastic lesions, foci of γ-glutamyltranspeptidase (GGTase)-positive hepatocytes, as the endpoint. To evaluate the tumor-promoting activity of phenobarbital, rats were administered diethylnitrosamine (DENA), 2.0 mmole/kg, followed by 500 ppm phenobarbital in their drinking water. After 6 weeks of phenobarbital promotion, the rats had an increased incidence of foci as compared to nonphenobarbital-treated rats. By 50 weeks, the number of foci in the nonpromoted animals equaled the number observed with promotion. The stability and progression of GGTase-positive foci was determined in rats that received a 2/3 partial hepatectomy, followed 24 hours later by DENA administration (0.3 mmole/kg). The rats then received 500 ppm phenobarbital in the drinking water for 7 weeks. After 7 weeks, half of the rats were continued on phenobarbital and the other half were removed from phenobarbital treatment. The number of foci observed in rats continued on phenobarbital treatment leveled off after 10 weeks of promotion, while in rats taken off phenobarbital it did not regress but increased at a slower rate, and, by 56 weeks, approached the number observed in rats subjected to continuous promotion. At 56 weeks, the size of foci was larger after continuous promotion. At 81 weeks, all 6 (100%) of the rats on continuous promotion had liver tumors, while only 3 of 6 (50%) of the rats removed from promotion had tumors. Promotion by phenobarbital stimulated the growth and decreased the time required for the appearance of GGTase-positive foci and liver tumors.

Introduction

Initiation/promotion was initially demonstrated in mouse skin as an increased yield of tumors resulting from repeated applications of croton oil to mice initiated with a single low does of benzo(a)pyrene (*1-3*). These studies resulted in the multistage theory of carcinogenesis, which has been now extended to other tissues, including the liver (*4, 5*). The first stage, initiation, includes the binding of the carcinogen to cellular DNA followed by fixation of the alteration during DNA replication. The altered genotype in the liver is expressed as cells with altered phenotypes. Cellular replication results in foci of hepatocytes possessing these altered activities.

Since the foci of altered hepatocytes are presumed to be early indicators of initiation in hepatocarcinogenesis (*6, 7*), they have been used as the end point in short-term assays for the detection of chemical carcinogens (*8-10*). These foci are readily identifiable histochemically as areas possessing either a decrease in glucose-6-phosphatase or adenosine triphosphatase or iron accumulation, or an increase in γ-glutamyltranspeptidase (GGTase) (*12-18*). In the present study, a focal increase of GGTase activity was used as the endpoint because: (a) approximately 90% of the foci stain positive for the enzyme (*19*), (b) GGTase is detectable histochemically in adult liver only in bile duct cells and in rats older than those used in this study (*20*) and (c) the enzyme is present in hyperplastic nodules and hepatocellular carcinomas (*21*). In this communication we describe the relationship of promotion to the incidence and stability of GGTase- positive foci.

Materials

Male Sprague-Dawley rats (Charles River Company, Portage, MI), weighing 175-200 g were used in these studies. The animals were maintained in accordance with accepted standards (*22*). They were fed Laboratory Chow (Ralston Purina Co., St. Louis, MO), and given drinking water *ad libitum*.

Diethylnitrosamine (DENA), reagent grade, was purchased from Eastman Organic Chemical Co. (Rochester, NY); sodium phenobarbital from Mallincrodt, Inc. (St. Louis, MO) or J. T. Baker (Glen Ellyn, IL); OCT compound and hematoxylin from Fisher Scientific Company (Pittsburgh, PA); and *N*-γ-L-glutamyl-4-methoxy-2-naphthylamine from Polysciences (Warrington, PA) and Bachem (Torrence, CA).

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Methods

Measurement of GGTase-Positive Foci

At sacrifice, the right lateral liver lobe was removed, cut into 10 × 10 × 2 mm blocks and frozen in OCT compound as previously described (*10*). The blocks were stored at −80°C until they were sectioned into 8 μm sections. These sections were stained for GGTase according to the procedure of Rutenburg et al. (*23*) and counterstained with hematoxylin. The sections were scanned, and foci with nine or more nuclei scored. Figure 1 depicts a typical GGTase-positve focus.

Experimental

Effect of Phenobarbital Promotion on the Number of GGTase-Positive Foci

Groups consisting of 10 male Sprague-Dawley rats each were dosed with 2.0 mmole/kg DENA by gavage, followed, 1 week after, by administration of 500 ppm of sodium phenobarbital in the drinking water. Two weeks after the DENA was administered, the rats were subjected to a 2/3 partial hepatectomy or a sham operation. Twenty-four hours after surgery, the rats were given 100 ppm phenobarbital in their drinking water. This reduction in the concentration of the phenobarbital was due to its increased toxicity in rats that received the partial hepatectomy. The concentration of phenobarbital was increased to 250 ppm at 4 weeks after partial hepatectomy.

Stability and Regression of GGTase-Positive Foci

Male Sprague-Dawley rats were given a partial hepatectomy followed 24 hr later by an intragastric administration of DENA (0.3 mmole/kg) in distilled water. One week later, all the rats were placed on 500 ppm sodium phenobarbital in their drinking water. Seven weeks later (8 weeks total), 20 rats were sacrificed, and only half of the remaining rats were continued on phenobarbital promotion. Ten rats from each group were sacrificed at 9, 11, 20 and 56 weeks, and six rats from each group at 81 weeks after DENA administration. Control rats, treated

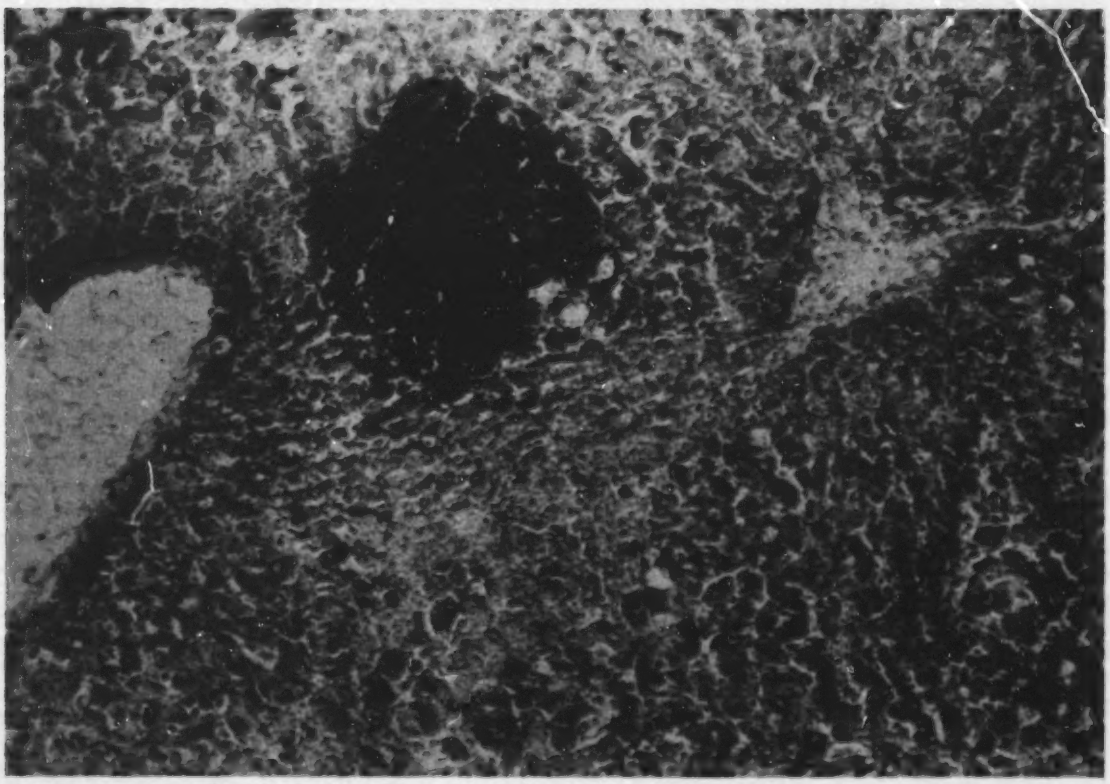

FIGURE 1. GGTase-positive foci. The liver section was stained for GGTase activity and counter-stained with hematoxylin. × 100.

with phenobarbital but not with DENA, were sacrificed at 8, 11, 20, 56 and 81 weeks.

Results

Effect of Phenobarbital Promotion on the Appearance of GGTase-Positive Foci

The results of the study comparing the effects of phenobarbital and of partial hepatectomy, on the incidence of DENA-initiated foci at 6 and 50 weeks, are shown in Table 1. After 6 weeks of promotion with phenobarbital and partial hepatectomy, the rats had an increased incidence of foci as compared to nonpromoted rats (Group 1 and 2). However, by 50 weeks the number of foci in the nonpromoted animals equalled the number observed with promotion. This indicated that the initiator determined the ultimate number of foci, while the promoter decreased the latent period required for development of the foci.

Stability and Progression of GGTase-positive Foci

The effects of continued versus discontinued promotion by phenobarbital on the incidence of GGTase-positive foci is shown in Figure 2. At 8 weeks, rats that received DENA administration followed by phenobarbital treatment had an increased incidence of GGTase-positive foci compared to rats

Table 1. Effect of phenobarbital on the incidence of GGTase-positive foci.[a]

GP	DENA (2 mmoles/kg)	Phenobarbital	Partial hepatectomy	GGTase foci/cm² of liver section[b] 6 wks post DENA	50 wks post DENA
1	+	+	+	19.8 ± 2.4 (5)	19.4 ± 1.45 (12)
2	+	-	-	8.3 ± 3.9 (5)	23.2 ± 6.72 (8)
3	-	+	+	0.8 ± 0.4 (4)	2.33 ± 0.44 (9)
4	-	-	-	0.2 ± 0.2 (4)	1.28 ± 0.62 (9)

[a]Data from Ford and Pereira (*10*).
[b]Means + standard error of the mean for number of animals in parenthesis.

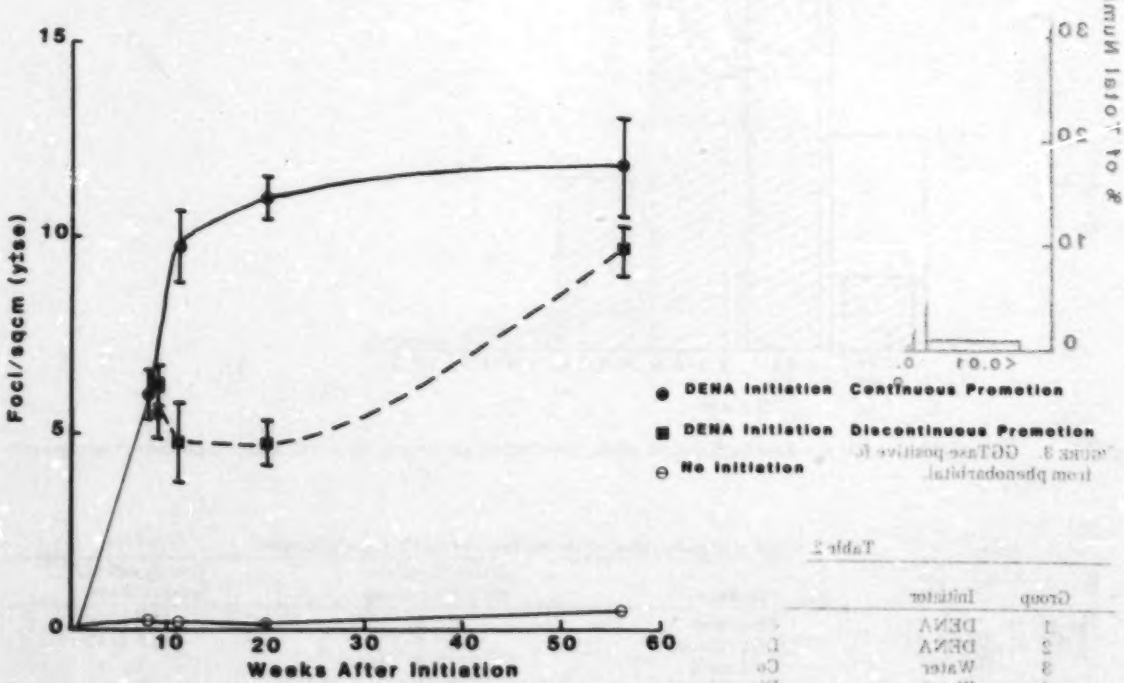

FIGURE 2. Stability of GGTase-positive foci with continuous vs. discontinuous promotion.

that received only phenobarbital. The number of foci observed in rats on continuous phenobarbital treatment leveled off after 10 weeks of treatment (week 11 of the experiment). In rats removed from phenobarbital at 8 weeks, the incidence of foci initially leveled off and then slowly increased so that, by 56 weeks, the incidence approached the level found in rats continued on phenobarbital.

At 56 weeks, the size of the foci in rats continued on phenobarbital was larger than that in the rats removed from phenobarbital (Fig. 3). The median size of the foci in rats continued on phenobarbital was 0.1-0.3 mm², while in rats whose treatment was terminated the median size was 0.03-0.1 mm² (this difference was statistically significant at $p < 0.001$). Foci larger than 1 mm² were found only in rats continued on treatment and represented 3% of the number of foci. Therefore, phenobarbital did not affect the ultimate incidence of DENA initiated foci, but stimulated the growth of the foci so that they could be observed earlier.

After 81 weeks, all six rats (100%) continued on phenobarbital had grossly visible liver tumors, while only three of the six rats (50%) which were removed from phenobarbital had tumors (Table 2). The rats continued on promotion had a total of 30 grossly visible tumors larger than the 3 mm diameter and the rats removed from promotion had 17 tumors. In rats not initiated with DENA, only one of nine rats which had received phenobarbital con-

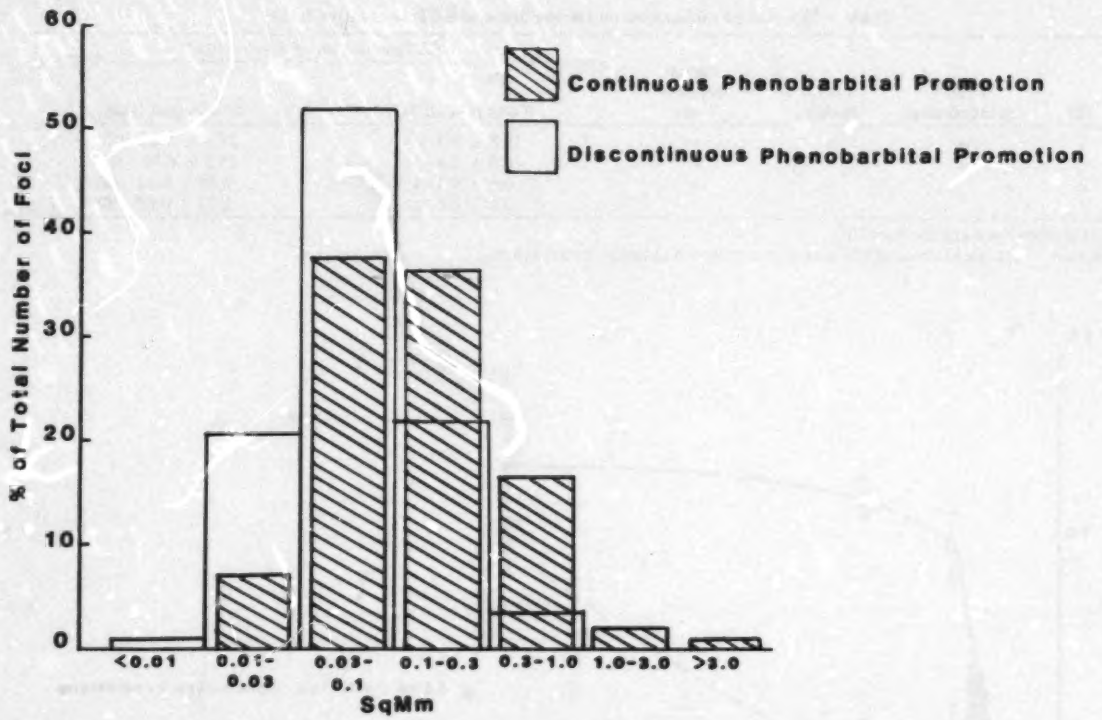

FIGURE 3. GGTase-positive foci size distribution at 56 weeks after DENA administration in rats either continued on or removed from phenobarbital.

Table 2. Effect of phenobarbital on the incidence of DENA-initiated tumors.

Group	Initiator	Promotion	Rats with tumors per number of rats	Grossly visible tumors (>3 mm)
1	DENA	Continued	6 / 6	30
2	DENA	Discontinued	3 / 6	17
3	Water	Continued	1 / 9	3
4	Water	Discontinued	0 / 5	0

tinuously had tumors, and none of the five rats which had received phenobarbital for only the initial 7 weeks had tumors.

Discussion

Phenobarbital, when administered to carcinogen initiated rats, has been shown to promote the induction of liver tumors (*24-26*) and to enhance the development of GGTase-positive foci (*10, 14, 16, 26-30*). The mechanism of phenobarbital enhancement of GGTase-positive foci was investigated in order to test its relationship to tumor promotion. Foci of GGTase-positive hepatocytes have been postulated to be the earliest observable evidence of initiation in the liver and their number to be a measure of the extent of the initiation of liver target cells (*11, 31, 32*). In the present studies phenobarbital decreased the time required for the appearance of DENA-initiated GGTase-positive foci by increasing their growth rate. However, the ultimate number of DENA initiated foci was independent of subsequent phenobarbital treatment. Kitagawa and Sugano (*26*) have also demonstrated that the appearance of foci occurred earlier in phenobarbital treated rats and that the incidence of foci was determined by the initiator. Our observations confirm that phenobarbital treatment, subsequent to DENA initiation, does not appear to alter the extent of initiation but acts to decrease the time required for the development of GGTase-positive foci and of tumors.

Tumors contain transformed cells that possess irreversible alterations in their phenotype. In the present studies, GGTase-positive foci were demonstrated to be a stable and irreversible lesion. Cessation of phenobarbital treatment during the time when foci were appearing did not result in regression of the foci. Actually, their incidence in rats whose treatment was discontinued did increase, though at a much slower rate, so that ultimately it did approach the incidence observed in rats previously treated with an initiator such as phenobarbital of the growth rate of established GGTase-positive foci was demonstrated by the larger size of the foci at 56 weeks in rats continued on phenobarbital compared to rats removed from phenobarbital. Therefore, phenobarbital continued to stimulate the growth of GGTase-positive foci after their appearance. When tumors began to appear at 81 weeks, the incidence was higher in rats continued on phenobarbital compared to those removed from phenobarbital. The precursor nature of GGTase-positive foci to hepatocellular carcinoma is supported by the fact that the foci are both stable and irreversible lesions, and that their growth is enhanced by phenobarbital.

The ability of a substance when administered to rats previously treated with an initiator such as DENA, to enhance the incidence of GGTase-positive foci and/or decrease the latent period for the appearance of foci has been purposed as a short-term screen for hepatic tumor promoters. The test substance in animals not initiated should have no effect on the incidence of foci. Substances that have been tested for their ability to enhance the incidence of carcinogen-initiated GGTase-positive foci and/or to promote tumor formation are presented in Table 3.

Table 3. Evaluation of chemicals as promoters of rat liver tumors.

Compound	Route	Endpoint	Result	References
Amobarbital	Diet	Tumors	Negative	(*33*)
	Water	Foci	Negative	(*34*)
Barbital	Water	Foci	Positive	(*34*)
5,7-Dibromo-8-hydroquinolone	Diet	Tumors	Negative	(*35*)
Dichlorodiphenyltrichloroethane (DDT)	Systemic	Tumors	Negative	(*36*)
	Diet	Tumors	Positive	(*33*)
Diphenylhydantoin	Diet	Tumors	Negative	(*33*)
DL-Ethionine	Diet	Tumors	Positive	(*37*)
Hexachlorobenzene	Diet	Foci	Positive	(*35*)
Hexobarbital	Water	Foci	Negative	(*34*)
8-Hydroxyquinoline	Diet	Tumors	Negative	(*37*)
Lindane	Diet	Foci	Positive	(*35*)
Mestranol	Diet	Foci	Positive	(*36*)
8-Nitroquinoline	Diet	Tumors	Negative	(*37*)
Norethynodrel	Diet	Foci	Negative	(*36*)
Pentabarbital	Water	Foci	Negative	(*34*)
Phenobarbital	Diet	Foci/tumors	Positive	(*24, 26-28, 33, 36*)
	Water	Foci/tumors	Positive	(*10, 29, 34, 38*)
Polychlorinated biphenyls	Diet	Foci/tumors	Positive	(*29, 38, 39*)
	Systemic	Foci/tumors	Positive	(*30*)
Quinoline	Diet	Tumors	Positive	(*37*)
2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD)	Systemic	Foci	Positive	(*40*)

Enhancers of the growth and incidence of GGTase-positive foci and/or tumor promoters in rat liver include drugs, hormones and environmental contaminants. A confounding effect in the evaluation of some of the enhancers of GGTase-positive foci was their induction in zone one (as defined by Rappaport) of GGTase activity (*41*). This zonal induction was observed in hexachlorbenzene-, lindane-, mestranol- and phenobarbital-treated rats and was of such a magnitude as to prevent the reading of some of the slides for the occurrence of foci. Where determined, there was a correlation between the ability of a substance to enhance foci formation and to promote tumor formation. This correlation was expected in view of the proposed precursor nature of the foci to hepatocellular carcinoma. In the presence of a promoter, maximum incidence of foci developed by 3 months which was much earlier than the appearance of tumors. Therefore, the ability of a substance to enhance the formation of GGTase- positive foci can be used as a short-term screen for liver tumor promoters. However, the confirmation that the substance which enhanced foci formation is a tumor promoter requires the actual demonstration of a decreased latent period for tumor promotion.

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